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14. ABSTRACT Chronic wasting disease (CWD) of deer and elk is unique among the transmissible spongiform encephalopathies. Our long-term goal is to better understand the epidemiology of CWD and thus develop strategies for management and control. The specific goals of these studies are to develop sensitive assays for PrPres as a marker for infectivity, and use these techniques to monitor the dynamics and modes of shedding of PrPres from orally infected mule and white-tailed deer and elk. Finally these techniques will be applied to investigating the nature of environmental contamination that may be associated with CWD transmission. Protease resistant prion protein from brains of CWD affected deer and elk (PrPres) and cellular PrPc were purified and used in a variety of detection assays. PrPres was detected using antibody based techniques which although substantially more sensitive than any current assay still need improvement. Deer and elk have been and infected orally to determine CWD shedding in vivo. In addition, in support of investigations of environmental contamination by the CWD agent, we have mapped areas of high, moderate, and low CWD contamination at two CWD endemic facilities and collected samples for assay to determine levels of the prion protein.					
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	20
Reportable Outcomes.....	20
Conclusions.....	21
References.....	21
Appendices.....	none

INTRODUCTION

Chronic wasting disease (CWD) of deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) is unique among the transmissible spongiform encephalopathies (TSEs) in that it occurs in free-ranging as well as captive wild ruminants and environmental contamination appears to play a significant role in maintenance of the disease. The precise modes of transmission of CWD are not known although we have shown that horizontal transmission and environmental contamination associated with excreta and carcasses may occur (Miller et al., 2004). But maternal transmission does not appear to play a significant role (Miller and Williams, 2003) in maintenance of CWD in cervid populations. Our long-term goal is to better understand the epidemiology of CWD and apply that information to development of strategies for management and control. To that end we are investigating the dynamics and modes of CWD agent shedding from infected mule deer, white-tailed deer, and elk. The approach includes experimentally infecting cervids, serial collections of a variety of biological samples, and assay of these materials by various means to attempt to detect protease resistant prion protein (PrP^{res}). In addition, because of the concern about environmental contamination associated with excreta, we will be collecting and assaying a variety of environmental specimens collected from areas of presumed high, moderate, and low contamination in CWD endemic facilities.

BODY

Aim 1: Develop analytical tools to detect PrP^{CWD} in excreta, blood, and environmental samples.

The Bio-Bar Code Assay (BBCA) for ultra-sensitive detection of proteins has been developed successfully in preclinical detection of important protein disease markers such as prostate specific antigen (PSA) and amyloid beta-derived diffusible ligand (ADDL) [1,2]. The bar code assay is an indirect sandwich immunoassay in which a protein is sandwiched between two antibodies where the primary antibody attached to a magnetic bead is used for target protein separation and where a probe coated with the secondary antibodies and bar code DNA is used for signal amplification[3]. The main technical hurdle in the bar code assay is to achieve a dose dependent, reproducible signal with a low background. Bao et al. show that DNA array hybridization with silver-amplified gold nanoparticles is the most effective mechanism reported to date for sensitive signal amplification while minimizing assay background [1]. Most other signal amplification mechanisms including PCR and colorimetric signal amplification mechanisms continue to suffer from high background which significantly affects the BBBCA's limit of detection (LOD) [1,3,4]. These recent advancements in BBBCA suggest that background issues associated with the signal amplification steps have been successfully addressed by changing the BBBCA secondary probe, and by minimizing nonspecific binding of barcode DNA by such steps as adding tRNA to the reactions [1]. Minimizing background levels, however, is still of critical importance in early steps of development especially in antibody pair selection. This report will address some of the important points for antibody selection and effective screening mechanisms for choosing effective antibody pairs.

Most bar code assays developed on important target proteins have a handful to a dozen commercial antibodies available for assay development thus limiting opportunities for broad analysis of antibody pairs (Nanosphere, Personal Communication). The prion protein (PrP) has

dozens of antibodies commercially and privately available and thus provides an opportunity to develop screening mechanisms and the opportunity to identify other important characteristics of antibody pairings in the bar code assay in order to reduce background. In this experiment magnetic beads (MB) were made per manufacturer's directions (Dynal) from seventeen monoclonal antibodies and twenty-eight monoclonal and polyclonal antibodies were biotinylated as the secondary antibody. A magnetic bead based ELISA as described by Bao et al. [1] provides the backbone for a preliminary screen. For each antibody pair, two reactions are performed with one reaction having no target PrP protein and the other reaction having target PrP protein at five ng/ml. All antibody pairs were tested including antibody pairs with overlapping epitopes and antibodies were tested against themselves. Next, the best performing antibody pairs, shown as either green or blue in figure 1, were screened in a secondary screen again using MB ELISA via a serial dilution of the target from five ng/ml down to fifty pg/ml. The BBCA was performed as described by Bao et al. [1] on the best performers from the previous screens. In the final BBCA screen the target protein amount ranged from 1 ng/ml to 5 pg/ml. The results from the secondary screen and BBCA are shown in figure 2.

The first and most important conclusion reached from the data shown in Figure 1 is that antibody pairs that work well in standard ELISA pairs do not necessarily work well in bead based ELISAs. In addition, one would assume that it would be fruitless to use antibody pairs with overlapping epitopes or using only one antibody as a pair; however, the data in this screen suggests otherwise as noted by good results for 5C4 on the MBs and 5C4 as the bAb. Other antibody pairs with overlapping epitopes support this conclusion as well (epitope maps for antibodies used are shown in supplemental data); nevertheless, the results may be the result of PrP and its propensity to form dimers thus exposing the same epitope twice. Extensive vortexing and sonication of the target recombinant PrP protein acquired from U.S. Biological should have minimized dimer formation. It is also interesting to note that reverse pairs often do not have similar performance. For example, with 3B6 on the magnet bead and 12F10 as the biotinylated antibody the screen shows that this pair is an excellent pair; however, the reverse pair where 12F10 is on the MB and 3B6 as the bAb is a low performer.

One logical hypothesis that is supported by the above observations is that the process of loading the Ab on the beads could change the kinetic binding properties of the Ab with its target, although, other changes in the assay also significantly affect assay performance include target protein buffer, amount of MB and bAb used per reaction, incubation times, and washing methods (supplementary data). Based on this information preparing MB and BBCA conditions in tightly controlled protocols thus becomes critical to maintaining assay consistency and in order to assess antibody pair kinetics. The summary statistics from the preliminary screen suggest that only ten percent show potential as good antibody pairs and about forty-four percent of the antibody pairs have high background; nevertheless, high background antibody pairs can show good results by varying BBCA reaction conditions (supplementary data).

The results from the secondary screen demonstrate that the preliminary screen can quickly identify good pairs with high sensitivity and acceptable assay performance as shown in the descriptive statistics in Figure 2. In the secondary screen only monoclonal antibody pairs were selected. Polyclonal antibodies should only be used when suitable monoclonals cannot be found. The variation in results as noted by data from the preliminary screen where R723 and R724 (fig.

1) have the same antigen in the same host species but significantly different results across the board which poses a problem with assay reproducibility over time. The normal dynamic range of a bead based ELISA is two logs [1] which indicates that the summary statistics for the secondary screen could be improved with a tighter control of the dynamic range especially the slope and R^2 values. False positives from the preliminary screen were about five percent but were concentrated in a couple of MB suggesting the possibility that MB stability or preparation are critical as noted above. From the screens, a single pair was identified for BBCA with the results of the BBCA assay shown in figure 2. This screening method does not preclude finding better performing pairs; this screening method merely identifies good pairs without the necessity of screening via the more time intensive and more expensive BBCA.

In conclusion, the MB ELISA can be performed simultaneously and as a screen with the BBCA to provide validation of the BBCA results. While antibody kinetics in other immunoassays for a specific antibody especially plate based ELISAs may be well characterized, bead-based, sandwich immunoassays antibody kinetics are often surprisingly different. Results reported here demonstrate again that array-hybridized, silver-amplified gold nanoparticles produce ultrasensitive, robust, reliable and reproducible results for detecting low abundance proteins. Finally, we report an assay for ultrasensitive detection of the normal prion protein which provides a foundation for the development of an ultrasensitive assay for the abnormal prion protein.

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Fig. 1. Preliminary Bead ELISA Screen Results. Bead-based ELISA were performed on recombinant deer PrP at five ng/ml or no target. ELISA results with significant background (Signal greater than .1 with no target) are represented by purple values. ELISA results with no significant signal are represented in red. ELISA results with low signal (target with signal > .1) are represented in yellow. ELISA results with moderate to high signal (> .25 and > .5) are represented in blue and green respectively. All assays were performed at least twice with MB concentration at 2.5 ug per reaction, with 25 ng per reaction, and with one hour incubation time. Antibody numbers in the columns correspond to those in the rows.

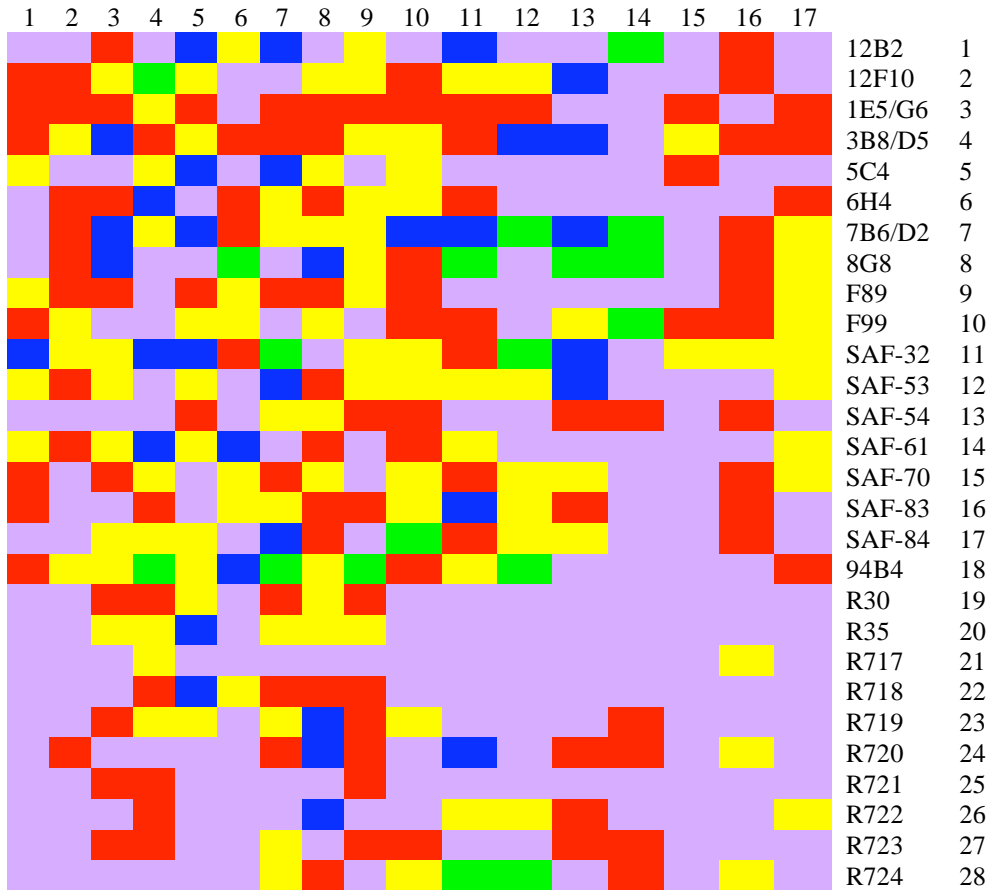
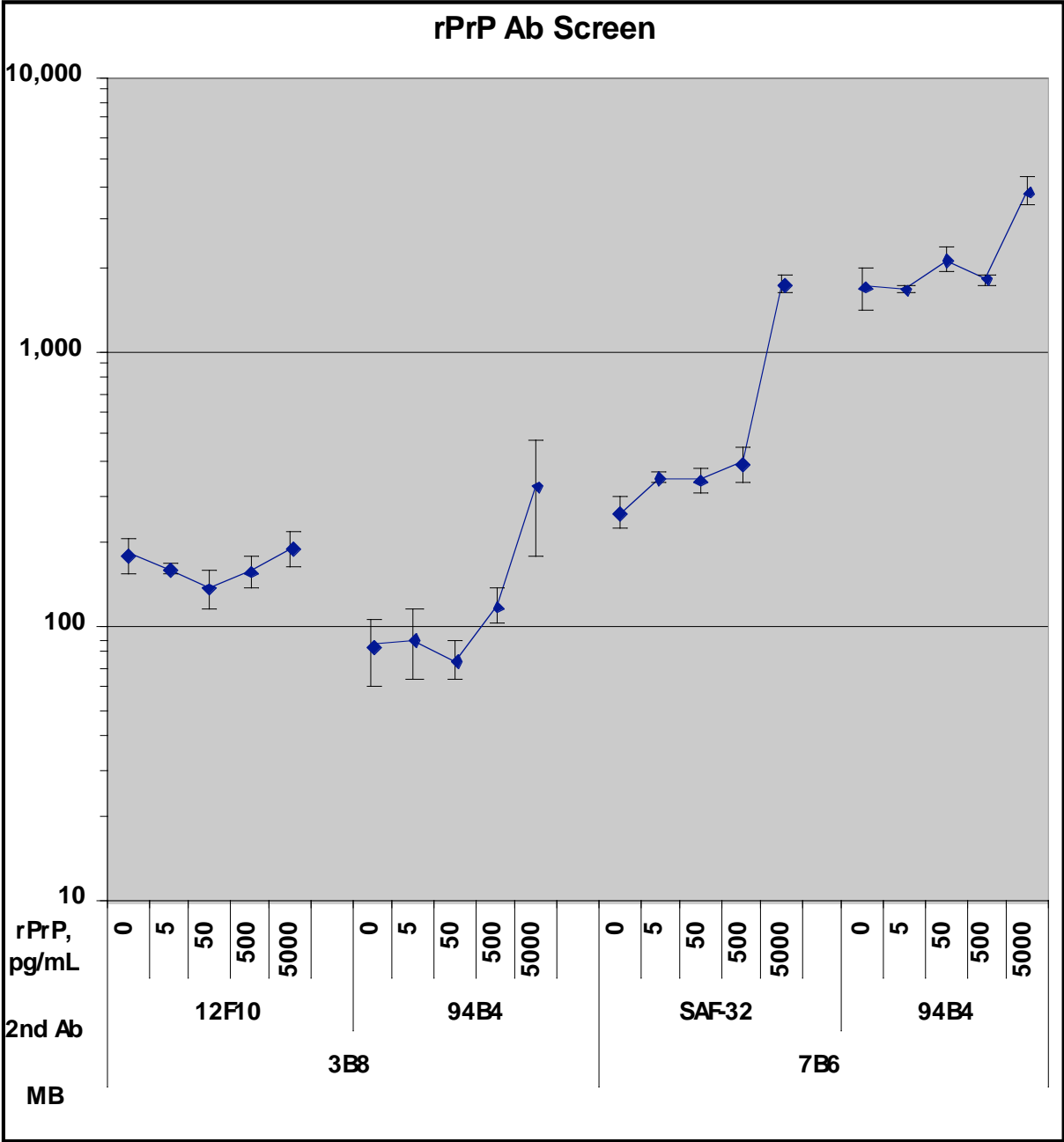


Fig. 2. Secondary Bead ELISA Screen Results and Bio-Bar Code Assay Results. (A) Bead-based ELISA were performed on recombinant deer PrP at titrated amounts of rPrP from five ng/ml to no target. (B) Initial results from bio-bar code assay using best pairs from the bead-based ELISA results.

(A)

Beads	bAb	5ng/ml	1ng/ml	500pg/ml	100pg/ml	50pg/ml	10pg/ml	NC	LOD	CV	R2	Slope
3B8	12F10	2.618	0.630	0.377	0.145	0.128	0.021	-0.008	12.168	43.98%	0.936	0.407
3B8	94B4	2.455	1.401	0.733	0.342	0.155	0.191	-0.034	160.646	61.07%	0.835	0.519
6H4	8G8	1.290	0.158	0.057	-0.045	0.039	-0.055	0.019	938.518	259.80%	0.938	0.262
7B6	94B4	2.394	1.170	0.897	0.232	0.188	0.145	-0.006	68.848	95.71%	0.763	0.447
7B6	SAF-32	2.292	1.007	0.591	0.123	0.067	0.028	-0.003	87.527	176.61%	0.761	0.445
F89	94B4	0.001	0.010	0.007	0.009	0.036	0.011	0.000	-1772.593	301.20%	0.029	-0.002
F89	SAF-84	0.032	0.007	0.310	0.009	0.020	-0.003	0.003	53843.593	233.77%	0.000	0.000
SAF-32	8G8	1.948	0.390	0.288	0.061	0.152	-0.011	0.009	185.732	119.05%	0.768	0.382
SAF-53	8G8	0.446	1.132	0.677	0.990	0.731	1.118	0.837	-9055.691	45.06%	0.098	0.000
SAF-53	94B4	0.197	0.221	0.133	0.149	0.122	0.059	0.173	12585.062	38.69%	0.052	0.000
SAF-53	SAF-32	0.117	0.118	0.109	0.056	0.067	0.063	0.053	2854.703	41.12%	0.118	0.000
SAF-54	8G8	0.259	0.303	0.242	0.271	0.269	0.196	0.275	3884.988	49.52%	0.151	0.000
SAF-61	12B2	0.343	0.434	0.281	0.326	0.312	0.289	0.321	20978.908	36.07%	0.015	0.009
SAF-61	7B6	1.974	0.648	0.407	0.148	0.126	0.104	0.108	535.150	67.89%	0.717	0.373
SAF-61	8G8	0.931	1.120	1.031	0.917	0.716	0.569	0.668	9256.405	38.07%	0.031	0.038
SAF-61	F99	0.104	0.083	0.126	0.082	0.085	0.081	0.082	31557.462	38.23%	0.018	0.003

(B)



Aim 2. Evaluate multiple biological samples collected from experimentally infected mule deer, white-tailed deer, and elk throughout the CWD incubation period.

Colorado: We continued sampling experimentally infected mule deer (*Odocoileus hemionus*) and white-tailed deer (*O. virginianus*) at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (FWRF, Fort Collins, Colorado) in support of Aim 2. During Sep 2005–Aug 2006, we collected urine, feces, saliva, and blood about every 42 days from surviving individual deer that were inoculated in December 2004 according to established study protocols. Samples have been sorted and stored at –20 C pending successful development and availability of assays under investigation (see Aim 1).

To confirm the infection status of inoculated deer, we collected tonsil biopsies (Wolfe et al. 2002) and evaluated them by immunohistochemistry (IHC) (Miller & Williams 2002) at 252 days post inoculation (dpi). We observed CWD-associated prion protein (PrP^{CWD}) deposits in tonsil biopsies from all 20 mule deer sampled; all of these deer were homozygous for serine [S] at codon 225 of the native prion protein (denoted as 225SS). Among the 21 white-tailed deer sampled, the presence of glycine [G] or S at codon 96 of the native prion protein (denoted as 96GG, 96GS, or 96SS) appeared to influence PrP^{CWD} deposition: ten of 11 96GG individuals and six of seven 96GS individuals had PrP^{CWD} in tonsil at 252 dpi; in contrast, none of three 96SS individuals showed PrP^{CWD} in tonsil at 252 dpi. When sampled again 342 dpi, the remaining 96GG and 96GS deer had PrP^{CWD} in tonsil, but the three 96SS deer remained negative. All three 96SS deer showed small amounts of PrP^{CWD} in tonsil when sampled 477 dpi. This apparent genetic influence on PrP^{CWD} deposition patterns is similar to those observations previously noted in mule deer (Fox et al. 2006) and white-tailed deer (M. W. Miller, unpublished data). The genetic variation in infected white-tailed deer under study here will afford an opportunity to also evaluate the influence of genotype on agent shedding once tools have been developed to make such assessments.

Eleven of 20 inoculated mule deer and 16 of 21 inoculated white-tailed deer surviving beyond 252 dpi remained alive as of the report date (29 August 2006; 617 dpi). All 11 surviving mule deer were showing clinical signs of CWD as of 617 dpi. Of the nine mule deer that died between 252 and 617 dpi, at least four died or were euthanized as a result of clinical CWD 491–617 dpi; whether CWD infection also contributed to the demise of the other five remains unclear. In a previous study (Fox et al. 2006), orally inoculated mule deer surviving >603 dpi (n = 5) died or were euthanized in end-stage clinical CWD 630–785 dpi; the somewhat shorter survival times encountered thus far in our study are most likely a result of our larger sample sizes and our use of more conservative criteria for determining the end-point of clinical disease to minimize suffering in subject animals. In contrast to mule deer, inoculated white-tailed deer typically have not shown obvious signs of CWD until shortly before their death. Of the five white-tailed deer that died between 252 and 617 dpi, all were 96GG. At least three of these died or were euthanized as a result of clinical CWD 517–616 dpi; whether CWD infection also contributed to the demise of the other two remains unclear.

In conjunction with our work to confirm infection status in inoculated subject deer, we conducted an ancillary study to assess the utility of rectal lymphoid sampling to diagnose CWD infections in mule deer and white-tailed deer (Wolfe et al., in review). We observed PrP^{CWD}

deposits in rectal mucosa from all 19 successfully sampled mule deer (all 225SS) by 381 dpi (Fig. 4). Similarly, 19 of 20 naturally infected mule deer sampled in the course of other ongoing studies had PrP^{CWD} in rectal mucosa. In white-tailed deer, presence of G or S at codon 96 also appeared to influence PrP^{CWD} deposition in rectal mucosa: nine of 11 infected 96GG individuals had PrP^{CWD} in rectal mucosa by 342 dpi; in contrast, only three of seven infected 96GS individuals had PrP^{CWD} in rectal mucosa by 381 dpi and none of three 96SS individuals showed PrP^{CWD} in either tonsil or rectal mucosa (Fig. ____). Our findings support further evaluation of rectal mucosa sampling in CWD surveillance programs.

Wyoming:

SAMPLING

During this period samples were collected from 29 CWD inoculated elk that had been divided into 3 groups (n = 10, 9, 10). Each group was sampled a total of 8 times at approximately 6 week intervals during this period. Samples collected included blood serum, saliva, urine, and feces. Samples were transported to the Wyoming State Veterinary Laboratory, Laramie, and banked. On February 9 bulls were electro-ejaculated to collect semen samples.

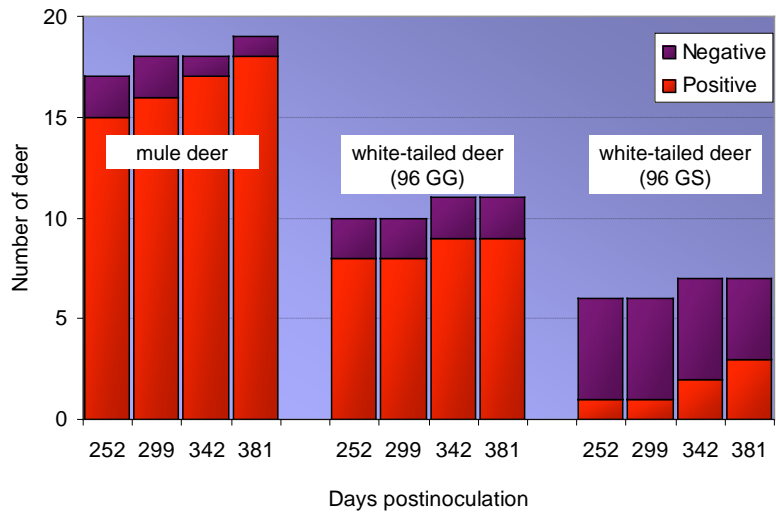


Figure 4. Cumulative proportion of tonsil biopsy-positive mule deer and white-tailed deer that also had deposits of PrP^{CWD} in lymphoid follicles of rectal mucosa tissue when sampled 252–381 days after oral inoculation. There generally appeared to be good agreement between immunohistochemistry results for tonsil and rectal mucosa samples for both deer species, and agreement improved over time. However, PrP genotype (encoding combinations of glycine [G] and serine [S] at codon 96, denoted as 96GG or 96GS) appeared to influence patterns of PrP^{CWD} deposition in white-tailed deer; all mule deer sampled were homozygous for S at codon 225, but similar patterns would be expected in this species as well.

MORTALITIES

During this period, 6 elk died and their carcasses were submitted to the Wyoming State Veterinary laboratory for necropsy. All of the elk that died were dam raised at the Thorne/Williams Wildlife Research Center and 5 of the 6 exhibited clinical signs of CWD prior to their deaths. There were no deaths in the cohort that was brought from Jackson, Wyoming in March of 2005.

DEATH	ELK ID#	WSVL REPORT
16-Feb-06	501	?
22-Feb-06	511	?
7-Apr-06	505	?
1-Jun-06	504	?
25-Jun-06	515	?
9-Jul-06	508	?

PREGNANCY

Blood samples were collected in May from the cows and sent to BioTracking LLC, (Moscow, Idaho) to determine pregnancy status. Results indicated 10 of the 19 cows were pregnant at that time. Two (2) cows calved during this period. Both calves were euthanized immediately and sent to the Wyoming State Veterinary laboratory for necropsy. No placenta was recovered from either cow.

BIRTH	ELK ID#	WSVL REPORT
10-Jun-06	517	?
12-Jul-06	520	?

OTHER

In November several elk in a nearby corral died from *Pasteurella multocida* infections. Several elk in this study group appeared depressed and possibly sick during this event. As a precautionary measure, all of the elk in this study group were treated with Nuflor (Schering-Plough Animal Health, Union, New Jersey). The sampling events that were scheduled in December were postponed until January to reduced stress and allow for recovery. No deaths were attributed to this event.

Here is the summary all animals from the past year. The first column represents animals we possessed thru Dec. 2005

Elk (<i>Cervus elaphus nelsoni</i>)	40
Mule deer (<i>Odocoileus hemionus</i>)	37
White-tailed deer (<i>Odocoileus virginianus</i>)	24
Deer mouse (<i>Peromyscus maniculatus</i>)	8
House mouse (<i>Mus musculus</i>)	3
Desert cottontail (<i>Sylvilagus audubonii</i>)	6
Ord's kangaroo rat (<i>Dipodomys ordii</i>)	1
Black-tailed prairie dog (<i>Cynomys leucurus</i>)	1
Thirteen-lined ground squirrel (<i>Spermophilus tridecemlineatus</i>)	1

This column represents animals we currently possess. (Includes Red Buttes, Sybille, and CDOW animals)

Elk (<i>Cervus elaphus nelsoni</i>)	31
Elk fetuses	6
Mule deer (<i>Odocoileus hemionus</i>)	23
White-tailed deer (<i>Odocoileus virginianus</i>)	25
Deer fetuses	22
Deer mouse (<i>Peromyscus maniculatus</i>)	23
House mouse (<i>Mus musculus</i>)	8
Desert cottontail (<i>Sylvilagus audubonii</i>)	11
Ord's kangaroo rat (<i>Dipodomys ordii</i>)	1
Black-tailed prairie dog (<i>Cynomys leucurus</i>)	2
Thirteen-lined ground squirrel (<i>Spermophilus tridecemlineatus</i>)	1

***Tables don't include insect numbers from either trapping site or small mammal numbers trapped at CDOW.

Aim 3. The goal of this Aim is to determine if PrP^{res} can be detected in samples collected from facilities contaminated with the CWD agent.

Both CWD endemic research facilities, FWRF and Sybille, have been qualitatively evaluated based on history and pen and pasture usage by CWD-affected animals and categorized into areas regarded as having low, moderate, or high probability of surface contamination with CWD agent (see previous annual reports for details).

Colorado:

At FWRF, we designed a replicated transect system to collect soil, vegetation, and invertebrate samples from areas regarded as having low, moderate, or high probability of surface contamination with CWD agent (Fig. 5) to support the goals of this Aim. We then collected samples in OctNov 2005 and MayJul 2006 using established protocols. For invertebrates, each "collection" represented one trap-day in the foregoing sampling scheme. In all, we made 175 fall collections (low = 77; moderate = 50; high = 46) and 365 spring collections (low = 117; moderate = 97; high = 151) of mixed invertebrates from our established transects. Sample collections have been stored at 20 C pending ongoing sorting and species identification of collected invertebrates. Sorted samples will remain frozen pending successful development and availability of assays under investigation (see Aim 1). In addition, small mammals have been sampled opportunistically in conjunction with other research studies and facility operations and tissues have been archived pending analysis.



Figure 5. Qualitative assessment of areas of low, moderate, and high CWD-associated surface contamination at the Colorado Division of Wildlife's Foothills Wildlife Research Facility, Fort Collins, Colorado.

Since the discovery of Chronic Wasting Disease (CWD) in the early 1980's [1, 2] the need for both an ultrasensitive and quantitative diagnostic assay for the prion protein to understand transmission mechanism(s) of CWD has become necessary. Without such an assay, research cannot proceed allowing for the development wildlife management plans to minimize the spread of CWD. The need for such an ultrasensitive assay is reinforced by the need to understand disease pathology to minimize the possibility of the disease overcoming the species barrier, thus becoming a threat to humans and animals who consume cervid tissues. Variant Creutzfeldt-Jakob (vCJD) disease, a human transmissible spongiform encephalopathy, is hypothesized to be caused by eating tissues from Bovine Spongiform Encephalopathy or BSE infected animals [3]. However, evidence from Hoover et. al. showing that the prion protein is found in skeletal muscle [4] coupled with earlier work from Williams et al. suggesting that the prion protein associated with CWD is at least as likely to cross the species barrier as BSE [5], suggests that CWD poses a risk to the food supply.

The foregoing observations describe the need for an ultrasensitive assay for the infectious form of the prion protein in cervids near the time of infection in order to understand CWD transmission mechanisms. These requirements obviously demand an extremely sensitive assay specific for the infectious form of the prion protein in various body fluids. Several possible body fluids could act as transmission mechanisms for CWD including urine, feces, saliva, as well as other tissues [6]. Although feces remains the most likely transmission mechanism candidate (E.W. Williams, unpublished data), urine is the most easily tested of the body fluids. Early reports by Shaked et. al. suggested discovery of an assay in urine which showed early detection of the protease-resistant PrP molecule of Syrian hamsters, cattle, and humans with prion diseases [7]. The evidence, however, was later disproved by Pruisner et. al. [8], showing that the

immunoblots detected antibody fragments rather than the prion protein, and by Niwa et. al., showing that bacterial contaminants led to false positive results [9].

With necessary controls taken in order to minimize the past mistakes associated with PrP discovery in urine, we characterize urine collected from preinfected and clinical animals using tightly controlled immunoassays to show that a protease-sensitive isoform of PrP, hereby denominated PrP_{sen}, can be detected in the urine of CWD-infected deer. These results lay groundwork for understanding the possible transmission of CWD through urine. In addition, a proteomics approach was taken in order to see if marker proteins are identifiable in order to develop a quick test.

A proteomic study was conducted of deer urine utilizing the Beckman Proteolab PF2D system. Samples were run on the PF2D followed by a tryptic digest and MALDI-TOF identification. Initial samples run indicated that sex hormones were the primary difference as the first sample run preinfection was a male deer and the CWD positive sample was a female deer. As a result, all future work immunoblot and proteomic work was done with samples separated and prepared by sex in pools. In order to correlate past studies conducted on other host systems, a immunoblot and silver stain of mule deer urine concentrated ten times was performed with both preinfection and CWD positive samples as well as male and female. In addition samples were digested with proteinase K for identification of PrP_{res}. An immunoblot protocol was developed that does not require a secondary antibody in order to address concerns raised by Prusiner that the secondary antibody cross reacted with IgG that resulted in false positive results. This work is shown in Figure 1 and corresponds to what was found by Prusiner, i.e. Immunoblots are not sensitive enough to identify PrP_{res} in urine, even though silver staining shows similarly sized proteins.

An immunoblot for PrP is usually sensitive at tens of nanograms using 12B2 antibody (Greg Raymond, Rocky Mountain Laboratory of the NIH, personal communication). Initial screening results of a bead based ELISA developed by Nanosphere using purified PrP_{sen} indicated sensitivity of the assay down to the tens of picograms per milliliter range. Based on this initial data, a screen of antibody pairs was conducted (data not shown) and several pairs of antibodies in the bead based ELISA showed high sensitivity in urine. Further titration experiments were conducted with the best performing antibody pairs. Urine was diluted ten fold in PBS in order to maintain antibody kinetic properties. A part of the sample was digested using standard proteinase K digestion protocols until these samples showed only background signal levels using the bead based ELISA. Recombinant PrP_{sen} was spiked back into the digested samples in a titration curve and run on the bead based ELISA in order to quantitate PrP_{sen} levels. Data indicates that PrP_{sen} in the urine at levels around 15-75 ng/mL. Data supporting these numbers are shown in Figure 2. It is important to note that three antibody pairs with five different epitopes support this conclusion. In addition, studies on human indicate that PrP_{sen} is found in plasma at hundreds of ng/mL levels (Phil Lefevbre, Nanosphere, Inc. personal communication).

As outlined earlier, a second proteomic study utilizing the Beckman ProteoLab PF2D was conducted, however, sample pools were segregated by sex in the second study. Data from the PF2D is shown in Figure 3. Samples are presently being identified using tryptic digests, mass spectrometry, and bioinformatic database analysis with results expected shortly.

Future Work

In order to finalize analysis of the prion protein in urine, antibody pairs in the bead based ELISA will be screened in diluted urine against the infectious isoform of PrP in similar experiments as were conducted using the noninfectious form of PrP. For additional sensitivity, bio-bar code assay experiments will likely be necessary to achieve the desired sensitivity. In addition antibodies from Anthony Williamson's group at Scripps that are specific for the infectious form of PrP have been acquired and will be tested in order to see if digestion can be eliminated.

Other transmission mechanism fluids such as feces and saliva will also be tested using bead based ELISA, bio-bar code assays and the PF2D proteomic system. Currently, experiments are being done in feces to determine optimal resuspension of the prion protein from feces. Saliva experiments will commence after completion of urine and feces studies.

Figure 1. Silver Stain and Immunoblot of ten times concentrated mule deer urine. (A) Silver stain gel of ten times concentration urine. (B) Immunoblot of ten times concentrated mule deer urine. These results support previous findings that the sensitivity of these methods is not high enough to detect PrP in urine.

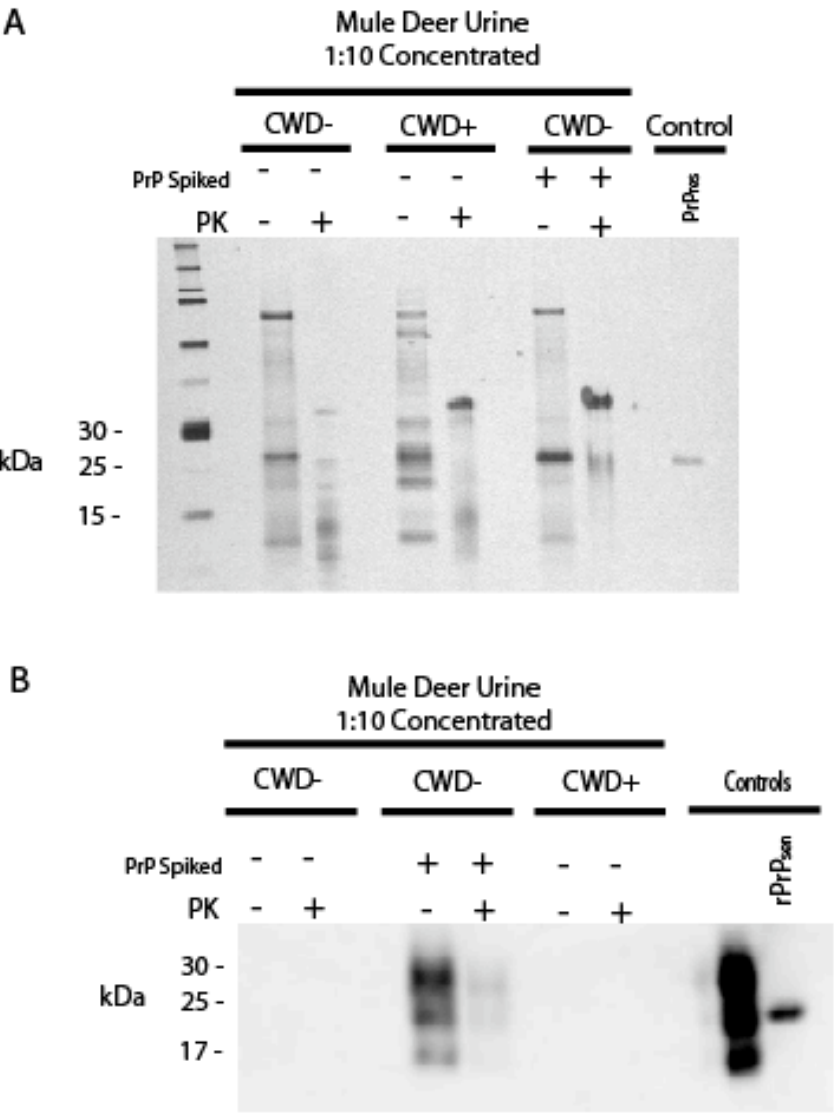


Figure 2. Bead based ELISA of PrPsen in Deer Urine. (A) Bead based ELISA of PrPsen in Deer Urine using 7B6 magnetic beads with SAF61 biotinylated antibody. (B) Bead based ELISA of PrPsen in Deer Urine using 7B6 magnetic beads with SAF53 biotinylated antibody. (C) Bead based ELISA of PrPsen in Deer Urine using 3B8 magnetic beads with 94B4 biotinylated antibody. (D) Bead based ELISA of PrPsen in Deer Urine using 7B6 magnetic beads with 94B4 biotinylated antibody. Results indicate PrPsen in urine at 15-75 ng/mL.

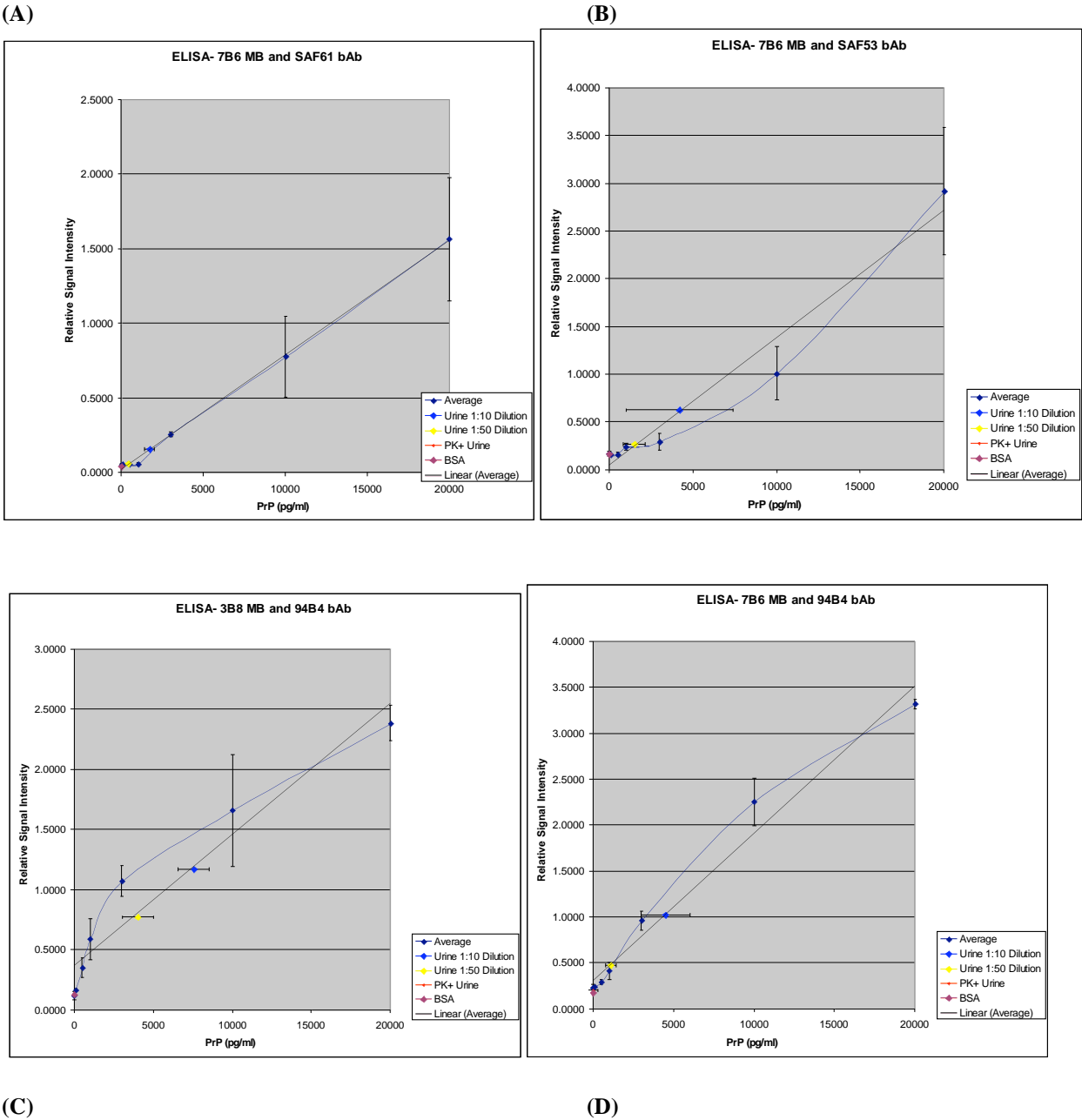


Figure 3. PF2D Deltavue of CWD- versus CWD+ mule deer urine. (A) PF2D Deltavue of CWD- versus CWD+ female urine. (B) PF2D Deltavue of CWD- versus CWD+ male urine. Green indicates higher amount in CWD+ samples whereas red indicates higher protein amounts in CWD- samples.

A.



B.



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KEY RESEARCH ACCOMPLISHMENTS

- Created both a bead based ELISA and bio-bar code assay for purified PrPsen as a proof of concept that can be applied to purified PrPres.
- Created a bead based ELISA that successfully determined the concentration of PrPsen in urine which will provide foundation for a more sensitive bio-bar code assay in urine and as a foundation for beads assays in other body fluids.
- Determined the proteome of Urine with easily identifiable protein difference that could potentially identify protein markers for a "quick field assay" for CWD in urine.
- Established automation for high throughput (96 samples in one hour) of samples in bead assays.
- CWD infections established and confirmed in mule deer and white-tailed deer.
- PrP^{CWD} demonstrated in tonsil and rectal mucosa biopsies from infected mule deer and white-tailed deer.

REPORTABLE OUTCOMES

- Demonstrating PrP^{CWD} in rectal mucosa from infected mule deer and white-tailed deer has applications to both antemortem and postmortem CWD diagnosis that may improve the efficacy and efficiency of ongoing surveillance programs nationwide.

PUBLICATIONS ARISING FROM GRANT WORK

Wolfe, L. L., T. R. Spraker, L. González, M. P. Dagleish, T. M. Sirochman, J. C. Brown, M. Jeffrey, & M. W. Miller. PrP^{CWD} in rectal lymphoid tissue of deer (*Odocoileus* spp.). *Journal of General Virology* (in review).

OTHER COLLABORATIONS ARISING FROM GRANT WORK

Surplus samples collected in the course of investigations supported by this grant have been shared with at least two other collaborating institutions (Rocky Mountain Laboratories, NIH-NIAID and Case Western Reserve University) in the hopes of advancing scientific understanding of CWD in particular and prion diseases in general. Other similar collaborative endeavors will be supported as feasible using materials arising from our work.

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CONCLUSIONS

We are still in the early stages of the proposed work primarily due to timing of the granting period relative to the biology of the deer and elk to be used in these studies. Progress is being made in purifying PrP^C and PrP^{res} from tissue and fecal samples but we have not yet been able to detect PrP^{res} from tissue, fecal, or environmental samples using MALDI-TOF, BBCA, western immunoblot, or ELISA. We still have not exhausted all the proposed approaches to preparation and concentration of samples for detection of PrP^{CWD}. As an alternative, as originally proposed, we are investigating use of transgenic mice expressing white-tailed deer PrP^C to assay samples for the presence of PrP^{CWD} through collaborative arrangements. Animal facilities and

other infrastructure are nearly completed and animals have been obtained so that CWD inoculations and sample collections will begin this fall.

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